



The Citrus Flavonoids Hesperetin and Nobiletin Differentially Regulate Low Density Lipoprotein Receptor Gene Transcription in HepG2 Liver Cells¹⁻³

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Abstract

Reduction of plasma cholesterol by citrus flavonoids is associated with effects on specific liver functions related to lipid handling. In previous *in vivo* studies, polymethoxylated flavones (PMF) reduced plasma cholesterol levels at lower doses than required for flavanones. To delineate hepatic mechanisms that underlie this differential potency, we used HepG2 cells to quantitate effects on expression of the LDL receptor (LDLR) gene. A dose-response analysis showed that 200 $\mu\text{mol/L}$ hesperetin, a flavanone present as a disaccharide in oranges, increased LDLR mRNA levels 3.6- to 4.7-fold of the untreated control. In contrast, nobiletin, a PMF found at the highest concentration in oranges and tangerines, achieved maximal stimulation of 1.5- to 1.6-fold of control at only 5 $\mu\text{mol/L}$. Transcriptional regulation of the LDLR gene by citrus flavonoids has been implicated but, to our knowledge, not directly demonstrated. Here, using transfection vector constructs containing the upstream region of the LDLR gene, we show differences in both potency and efficacy in the induction of transcription, with peak stimulation of 5.3- to 7.5-fold of control at 150–160 $\mu\text{mol/L}$ hesperetin and 3- to 3.8-fold of control at 10–20 $\mu\text{mol/L}$ nobiletin. Hesperetin sustains induction, whereas nobiletin is inhibitory at high doses, resulting in an inverted-U dose response. The sterol regulatory element (SRE) in the LDLR gene upstream region plays a crucial role, because mutation of this site strongly attenuated induction in response to hesperetin or nobiletin. Thus, citrus flavonoids are likely to act through the SRE-binding proteins, with PMF initially activating these mechanisms at considerably lower concentrations than flavanones. *J. Nutr.* 138: 1274–1281, 2008.

Introduction

The nonnutritive constituents of citrus fruits reduce several risk factors for cardiovascular disease in humans and animals. Ingestion of citrus fruits or juices decreases levels of plasma cholesterol and triglycerides (TG)⁷, liver cholesterol and cholesteryl esters, and the circulating LDL:HDL ratio (1–8). The bioactive compo-

nents in citrus include flavonoid compounds, which are present in thousands of different structural forms in a diversity of plants (9). The major citrus flavonoids include the flavanones, hesperetin and naringenin, and the polymethoxylated flavones (PMF), such as nobiletin and tangeretin (Fig. 1) (10). Oral administration of specific flavonoids decreases plasma cholesterol and/or TG levels in rabbits (11), rats (12,13), mice (14,15), and hamsters (16), with the PMF exhibiting greater potency than the flavanones in the hamster model. In humans, dietary supplementation with a flavanone lowers total and LDL cholesterol in the plasma (17) and recent clinical trials demonstrated that citrus PMF, in combination with palm tocotrienols, reduce plasma TG and total and LDL cholesterol (18). Because the liver plays a major role in cholesterol and lipid metabolism, studies have focused on the action of citrus flavonoids in this organ and have identified multiple effects on lipid-handling pathways in intact animals (11,13,15).

To analyze mechanisms of hepatic activity of flavonoids in greater detail than is possible in whole animals, the human HepG2 liver cell line has been extensively characterized. For example, using output of the protein apolipoprotein B (apoB) as an indicator of lipid secretion, it was shown that citrus

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³ Supplemental Table 1 and details of the protocols for RNA isolation, RNA gel electrophoresis and molecular hybridization, quantitative RT-PCR, transfection vector assembly, DNA purification, and transfections and luciferase assays are available with the online posting of this paper at jn.nutrition.org.

⁷ Abbreviations used: apoB, apolipoprotein B; DMSO, dimethyl sulfoxide; LDLR, LDL receptor; PI3K, phosphatidylinositol 3-kinase; PMF, polymethoxylated flavone; qRT-PCR, quantitative RT-PCR; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; TG, triglyceride.

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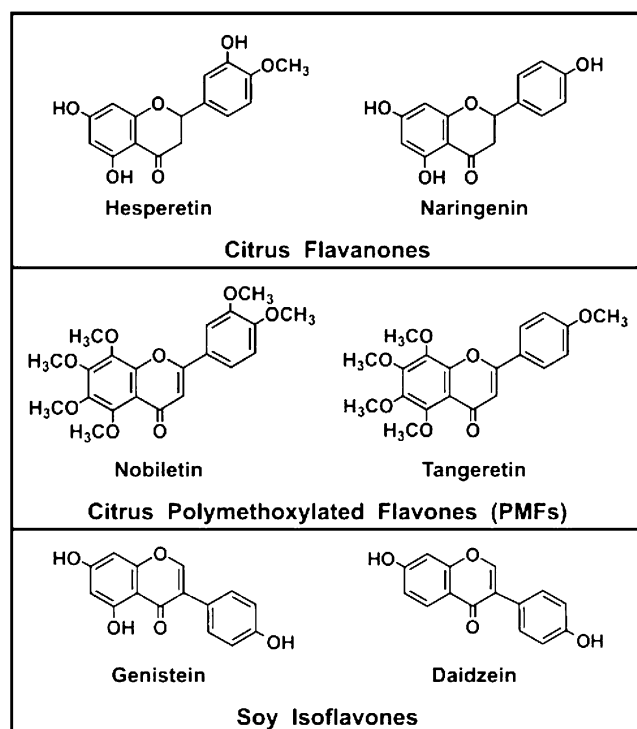


FIGURE 1 Structures of citrus and soy flavonoids.

flavonoids decrease the rate of apoB synthesis, secretion of newly synthesized apoB, and apoB mass in the medium (19–26). The reduction of apoB secretion from HepG2 cells occurs with lower concentrations of PMF compared with flavanones (22), analogous to the higher potency of the PMF in the hamster model (16). Flavonoid treatment also decreases the availability of lipids for export by inhibiting cholesteryl ester synthesis, microsomal TG transfer protein enzyme activity, and/or TG accumulation in the microsomal lumen (19–21,23–25).

In conjunction with establishing physiological parallels between intact liver and HepG2 cells, it is important to consider the type of flavonoids available to the liver *in vivo*. PMF, such as nobiletin and tangeretin, are present in the fruit as aglycones (10), allowing this form to be used both *in vivo* and in cultured liver cells (16,22,25). In contrast, the glycosylated flavanones, hesperidin and naringin, naturally occur in citrus and, consequently, are used in human and animal studies (11–17). However, deglycosylation takes place in the intestines before absorption into the circulation (27–30), warranting the administration of the aglycones, hesperetin and naringenin, to isolated liver cells (19–24,26).

In addition to inhibition of lipid export, effects of citrus flavanones on LDL receptor (LDLR) activity have been demonstrated through enhancement of binding, uptake, and degradation of LDL in HepG2 cells (20). Consistent with these stimulatory effects on the LDLR protein, hesperetin and naringenin act at the mRNA level to increase the concentration of LDLR mRNA (20). It is plausible that these effects on LDLR mRNA are due to regulation of gene transcription by sterol regulatory element (SRE)-binding proteins (SREBP), because the LDLR gene is a positive target of these transcription factors (31). In support of this mechanism, naringenin increases the mass of both nuclear and cytoplasmic SREBP-1 (24) and the soy isoflavones genistein and daidzein elevate mature SREBP-2 levels (32). We have found no studies that investigated the role of extended native DNA

sequences in the regulation of LDLR gene transcription by citrus flavonoids in liver cells. Thus, because the physiological relevance of citrus effects on LDLR are established and the LDLR gene is one of the most well-characterized SREBP target genes, we have examined the effects of nobiletin and hesperetin on both LDLR mRNA levels and gene transcription in HepG2 cells and have investigated the role of the SRE. We compared PMF to the flavanone to determine whether there were differences in potency, efficacy, and overall patterns of expression.

Materials and Methods

HepG2 cell culture. HepG2 cells from the American Type Culture Collection were maintained at 37°C, 5% CO₂ in Eagle's minimum essential medium with Earle's balanced salt solution and 2 mmol/L glutamine (GIBCO), 7–10% (v:v) fetal bovine serum (Atlanta Biologicals or US Biotech), 1 mmol/L sodium pyruvate (GIBCO), 0.1 mmol/L MEM nonessential amino acids (GIBCO), and 17 mg/L gentamicin sulfate (Sigma). Frozen stocks in the above medium containing 5% (v:v) dimethyl sulfoxide (DMSO) were prepared as recommended by the American Type Culture Collection and, to ensure uniformity, cells from a single frozen stock tube were maintained in culture for no longer than 10 wk. Uncoated T-75 flasks (Corning) were used for propagation of the cells for preparation of total cellular RNA (~25 × 10⁶ cells per flask at confluency) and 24-well Primaria microtiter plates (Falcon) were used for transfections.

Flavonoid treatment of HepG2 cells. Hesperetin (≥95% pure), a flavanone from oranges, and synthetic genistein (≥98% pure), a soy isoflavone, were obtained from Sigma. Nobiletin was purified from tangerine peel and recrystallized twice to yield a final purity of >99% (33). Concentrated 50 mmol/L flavonoid stock solutions were prepared in DMSO. The final concentration of DMSO introduced into the cultured cells, either with the flavonoids or as the vehicle in control cells, was ≤0.4% (v:v). To ensure that basal levels of SREBP were available to mediate flavonoid action, the cells were not preexposed to low sterol conditions, which would tend to mobilize SREBP stores, or to high sterol conditions, which would more strongly sequester SREBP in the endoplasmic reticulum (34).

Transfections. DNA vectors were introduced into the HepG2 cells by transfection with Lipofectamine Reagent (Invitrogen). To obviate well-to-well variation in transfection efficiency, we developed a batch-wise transfection procedure, which is described in more detail in the Online Supporting Material. In each experiment (*n*), there were 2 replicates for each flavonoid dose and 2–4 replicates for the untreated control.

Statistical methods. The *t* test was performed as described (35). For the experiments in Fig. 3C,D, Bartlett's test indicated equal variance and Dunnett's test was performed (35). For the experiments in Figs. 3B and 4, standard ANOVA followed by tests of normality and equal variance (36,37) of the residuals were conducted on natural log-transformed data. The overall F-test and Dunnett's test were then carried out. For Fig. 5A, the data from nobiletin-treated cells were analyzed as described in the preceding sentence, because the residuals from the log-transformed data were normal and had equal variances. The untransformed data from hesperetin-treated cells were analyzed using a mixed linear model approach, which allowed for unequal variances for each dose in this data set, followed by the overall F-test and Dunnett's test.

Because a more complex analysis was necessary for Fig. 5B, ANOVA with mixed effects and associated tests of contrasts were conducted. The mixed linear model was:

$$Ln(y_{ijkl}) = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \varepsilon_{ijkl},$$

where $Ln(y_{ijkl})$ is the natural log-transformed measure of luciferase expression for treatment *i* (none, hesperetin, or nobiletin), type *j* (wild type or mutant), run *k*, and replicate *l*. The fixed effects were treatment and type and the random effects were run (γ) and error (ε). The random terms were assumed to be uncorrelated random variables having 0 means

and variances σ_y^2 and σ_e^2 . This model was fit using SAS software (v9.1.3) and the GLIMMIX procedure. Estimation was carried out using restricted maximum likelihood and degrees of freedom were based on the Kenward-Roger method. Model diagnostics were thoroughly evaluated to ensure proper model fit and appropriateness of underlying theoretical assumptions (i.e. normality and unequal group variances).

Other experimental procedures. RNA was purified with the RNeasy system (Qiagen). RNA was size fractionated on a denaturing formaldehyde gel and transferred to GeneScreen (New England Nuclear/Perkin Elmer) for molecular hybridization. Quantitative RT-PCR (qRT-PCR) utilized SYBR Green chemistry and was run on the iQ5 RT-PCR Detection system (Bio-Rad). Luciferase assays were done with the Dual-Luciferase Reporter Assay system (Promega) (see **Online Supporting Material**).

Results

Identification of 3 size variants of LDLR mRNA induced by hesperetin in HepG2 cells. Three potential forms of human LDLR mRNA have been described (Fig. 2A): 1) the full-length molecule of 5175 bases, excluding the 3' polyA tail (GenBank accession no. NM_000527.2) (38); 2) a shorter RNA that uses a polyadenylation signal at nucleotides 4579–4584 to terminate

slightly downstream of position 4584 (GenBank accession no. NM_000527.2); and 3) a molecule ending at position 3521 (relative to the numbering in NM_000527.2), identified in Burkitt lymphoma (Genbank accession no. BC014514). (See Note Added in Proof for minor changes to RNA sizes.) Previous studies showed that 200 $\mu\text{mol/L}$ hesperetin induced LDLR mRNA levels in HepG2 cells, but the particular size variants were not identified (20). To determine which LDLR mRNA species were regulated by flavonoid treatment in HepG2 cells, we used a set of complementary probes that differentiated between the 3 potential mRNAs (**Supplemental Table 1**; Fig. 2A). We show by hybridization to size-fractionated RNA from HepG2 cells that Probe 3 detected a broad band at ~ 5000 bases, as well as a sharper band at ~ 3500 bases (Fig. 2B, lane 6). To clarify the identity of the broad band, we hybridized the RNA with either probe 1 that recognized the largest species of 5175 bases exclusively, or probe 2 that hybridized to both the 5175 and the 4584 base mRNAs (Fig. 2A). In lane 2 of Fig. 2B, probe 1 hybridized to RNA corresponding to the upper part of the broad band in lane 6, consistent with the full-length LDLR mRNA. Probe 2 hybridization was more diffuse, consistent with the detection of both the 5175 and the truncated species of 4584 bases (Fig. 2B, lane 4). Thus, we conclude that all 3 forms of LDLR mRNA are produced in HepG2 cells and induced by 200 $\mu\text{mol/L}$ hesperetin (compare lanes 2 to 1, 4 to 3, and 6 to 5). For subsequent investigations, we used probe 3, because it appeared to interact with the RNA more efficiently than the other 2 probes.

Dose-dependent regulation of LDLR mRNA levels by flavonoids of 3 structural classes. Citrus PMF are physiologically effective at lower doses than flavanones in reducing plasma cholesterol in animals (16) and, at the cellular level, in decreasing apoB secretion from HepG2 cells (22). Also, intermediate concentrations of genistein, a soy isoflavone, have been shown to inhibit apoB secretion and to increase LDLR mRNA amounts in HepG2 cells (39). Therefore, we compared the potency and efficacy of a PMF (nobiletin) to those of a flavanone (hesperetin) and an isoflavone (genistein) at the molecular level of LDLR gene expression. These representative compounds were chosen based upon higher activity compared with other members of their respective class (20,39, our unpublished data). For these experiments, HepG2 cells were treated with a concentration range of 5–200 $\mu\text{mol/L}$ hesperetin, 1–200 $\mu\text{mol/L}$ nobiletin, or 1–200 $\mu\text{mol/L}$ genistein and total RNA was purified 24 h later. No systematic differences were found in the total amount of RNA recovered from untreated vs. flavonoid-treated cells across the dose ranges tested (see **Online Supporting Material**), suggesting that the compounds did not cause considerable cell death. The RNA was analyzed for changes in LDLR mRNA levels both by hybridization of probe 3 to size-fractionated RNA to ensure the integrity of the LDLR mRNA and by qRT-PCR for more rapid quantitation of relative RNA amounts. Like the hybridization probe, the qRT-PCR primers detected all 3 isoforms of the LDLR mRNA (see **Supplemental Table 1**).

Representative hybridization data are shown from 1 experiment (Fig. 3A). For each sample, the LDLR mRNA was normalized to ribosomal protein S19 mRNA. Four independent experiments were performed with all 3 flavonoid treatments and the data from both hybridization and qRT-PCR are shown (Fig. 3B,C,D). The citrus flavanone, hesperetin, induced LDLR mRNA by 3.6- to 4.7-fold of control, with maximal stimulation occurring at 150–200 $\mu\text{mol/L}$ (Fig. 3B). Compared with the untreated control, all hesperetin doses ≥ 40 $\mu\text{mol/L}$ elevated LDLR mRNA levels ($P \leq 0.05$). The full dose-range response is

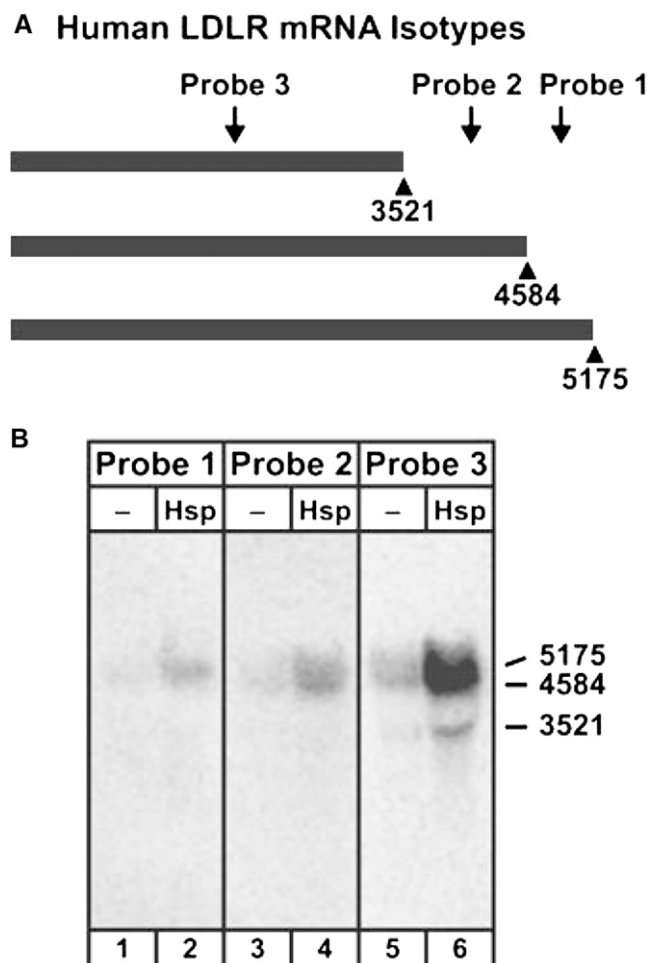


FIGURE 2 Schematic representation (A) and experimental detection (B) of 3 LDLR mRNA isotypes induced by hesperetin in HepG2 cells. Hybridization analyses were performed on size-fractionated total RNA from untreated and hesperetin-treated cells. The sizes of the 3 mRNA isotypes are denoted on the right.

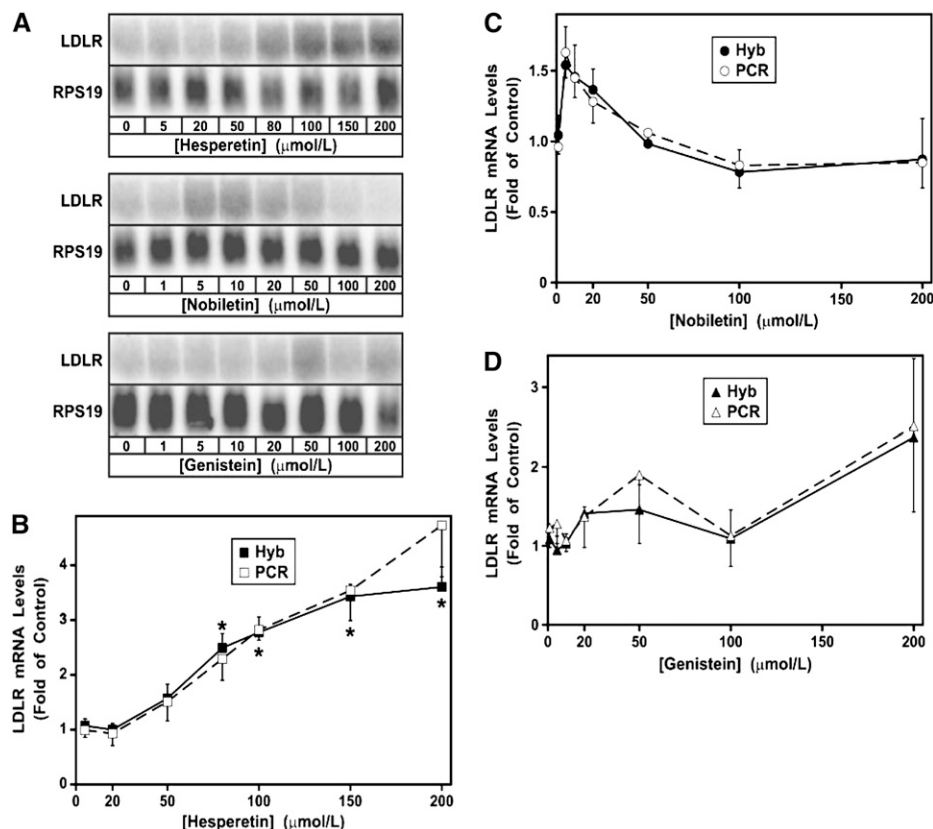


FIGURE 3 The impact of 24-h flavonoid treatment on LDLR mRNA levels shown by hybridization (A) and quantified by hybridization and qRT-PCR (B–D). Hybridizations were conducted using ribosomal protein S19 mRNA as an internal control. Both hybridization and qRT-PCR were used to corroborate the results from each method independently. Quantitation and normalization of LDLR mRNA levels from 4 separate experiments (except $n = 3$ for 2 nobiletin doses) were conducted for each technique. Error bars represent \pm SEM for the hybridizations and \pm SEM for the qRT-PCR data. *Hybridization data different from the untreated control, $P \leq 0.05$ (Dunnett's test).

consistent with previous results showing induction of 6.6-fold of control at 200 $\mu\text{mol/L}$ hesperetin (20). Genistein effects were quite variable over the dose range (Fig. 3D) and not significant at any dose by Dunnett's test ($P \leq 0.05$); however, other investigators have found that genistein induced LDLR mRNA in HepG2 cells by 3.1- and 5.7-fold of control at 50 and 100 $\mu\text{mol/L}$, respectively (39). In contrast to hesperetin, nobiletin maximally induced LDLR mRNA by a relatively modest 1.5- to 1.6-fold of control, but this stimulation occurred at 5–10 $\mu\text{mol/L}$, while higher concentrations were ineffective (Fig. 3C). When we compared the 10 $\mu\text{mol/L}$ nobiletin dose to the untreated control by t -test, the difference was significant at $P \leq 0.05$ but it was not when we used the correct and more stringent Dunnett's test for multiple comparisons (35). Nonetheless, because of the consistency of the pattern at several doses and the reproducibility of the results between experiments by 2 independent methods of quantitation, further investigation of the underlying molecular mechanisms was warranted.

Flavonoid effects on LDLR gene transcription. Because LDLR mRNA levels can be affected through changes in mRNA stabilization (40,41) and/or gene transcription, we used gene transfection assays to determine whether LDLR mRNA regulation was primarily due to effects on gene transcription. The greater sensitivity of this approach allows for a more thorough statistical evaluation to corroborate the dose response patterns observed at the mRNA level. The LDLR(–1480 to +35) luciferase construct, containing the regulatory region of the human LDLR gene spanning from 1480 nucleotides upstream to 35 nucleotides downstream of the transcription start site (position +1), was introduced into HepG2 cells, which were then treated with a dose range of the flavonoids for 24 h. In response to hesperetin, LDLR gene transcription increased

steadily until essentially reaching a plateau at $\sim 120 \mu\text{mol/L}$, with maximal induction of 7.5-fold of control (Fig. 4A). In contrast, nobiletin was effective at much lower doses, achieving maximal stimulation of 3.0-fold of control at 10 $\mu\text{mol/L}$ (Fig. 4B). However, the higher doses of 100 and 200 $\mu\text{mol/L}$ nobiletin were distinctly inhibitory in marked contrast to the sustained induction by hesperetin. This negative regulation is due to specific action on the LDLR gene, because normalization to the *Renilla* luciferase corrected for any general toxicity at higher flavonoid concentrations. The overall pattern of stimulation of LDLR gene transcription by genistein was similar to that of nobiletin, but maximal induction was only 1.8-fold of control at 10 $\mu\text{mol/L}$ (Fig. 4B) and negative effects at high doses were more pronounced. Our studies with genistein were undertaken initially to determine whether this well-known soy isoflavone is more effective than the citrus flavonoids in regulating LDLR gene expression. The variable responses at the mRNA level (Fig. 3D), together with the lower induction of gene transcription (Fig. 4B), show that the soy compound is not superior to the citrus flavonoids. Therefore, we continued to investigate the molecular mechanisms of hesperetin and nobiletin without further analysis of genistein.

To delimit the location of DNA regulatory elements required for flavonoid action, the transcription assay was next conducted with a construct containing only 142 bp of the upstream regulatory sequence, designated LDLR(–142 to +35). This segment contains an SRE sequence at –65 to –56 upstream of the transcription start site (31,42). Transcription from the LDLR(–142 to +35) construct rose gradually with increasing doses of hesperetin until leveling off above 150 $\mu\text{mol/L}$, achieving a maximal response of 5.3-fold of control (Fig. 5A). Thus, the –142 to +35 segment upstream of the LDLR gene is sufficient to mediate responsiveness to hesperetin. With nobiletin

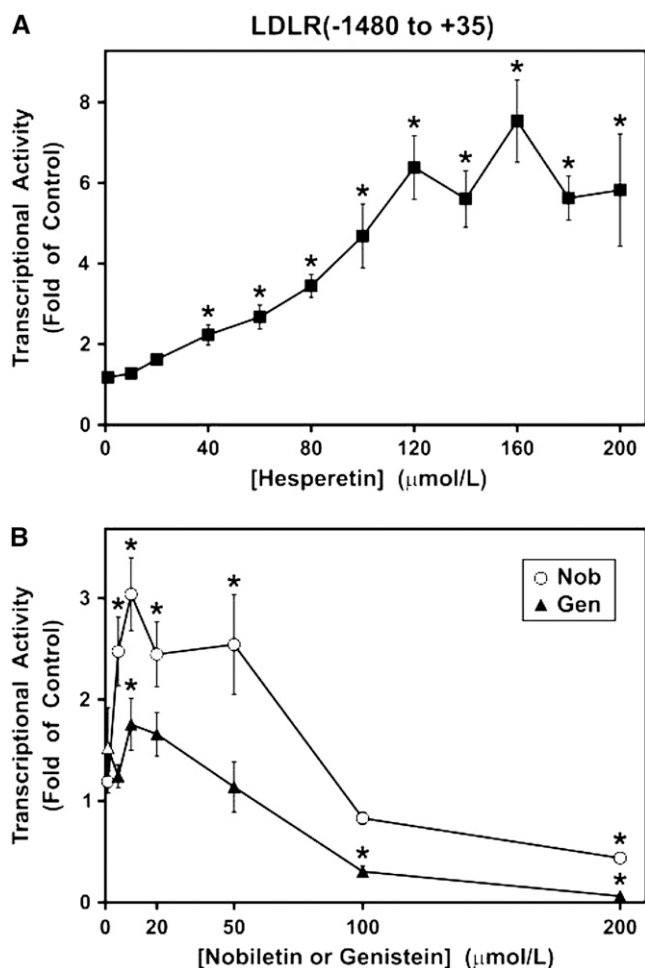


FIGURE 4 Dose effects of hesperetin (A) and nobiletin or genistein (B) on luciferase reporter vectors containing regulatory sequences of the human LDLR gene from -1480 to $+35$ relative to the transcription start site. Transfected HepG2 cells were treated with hesperetin ($n = 5$), nobiletin ($n = 5$), or genistein ($n = 3$) for 24 h over a range of concentrations. Luciferase values were normalized as described in the Online Supporting Material. Error bars are \pm SEM. *Different from the untreated control, $P \leq 0.05$ (Dunnett's test).

treatment, transcription from this DNA reached maximal induction of 3.8-fold of control at $20 \mu\text{mol/L}$, with repression at high doses (Fig. 5A), indicating that this DNA is sufficient not only for positive induction at low concentrations but also for inhibitory effects at high concentrations of nobiletin. The close agreement between the results in the mRNA and gene transcription assays indicates that transcriptional regulation is the major mechanism by which the citrus flavonoids control LDLR mRNA amounts.

Role of the SRE in flavonoid induction of LDLR gene transcription. The role of the SRE (ATCACCCAC) at -65 to -56 in mediating flavonoid regulation of the LDLR gene was examined by mutating position -59 (ATCACCGCAC), because this single point mutation disrupts modulation of LDLR gene transcription by SREBP (31,42). Transcription of the wild-type LDLR(-142 to $+35$) construct was stimulated 3.7-fold of control by $120 \mu\text{mol/L}$ hesperetin, whereas the responsiveness of the SRE mutant was attenuated to 1.6-fold (Fig. 5B). Likewise, the response to $10 \mu\text{mol/L}$ nobiletin markedly declined from 2.9-

fold of control for the wild type to 1.1-fold for the mutant. In addition, basal expression in the mutant was reduced to 23% of the wild-type level, but expression was still >27 -fold above the assay background.

Discussion

Role of the SRE in citrus flavonoid regulation of LDLR gene transcription and potential mechanisms of SREBP activation. The SRE at positions -65 to -56 (31,42) of the human LDLR gene regulatory region mediates induction of LDLR gene transcription by a number of stimuli, including low sterol concentrations, insulin, estradiol, and interleukin-6 (31,42–44). Furthermore, involvement of SREBP in flavonoid action in HepG2 cells has been suggested (24,32). Hence, we

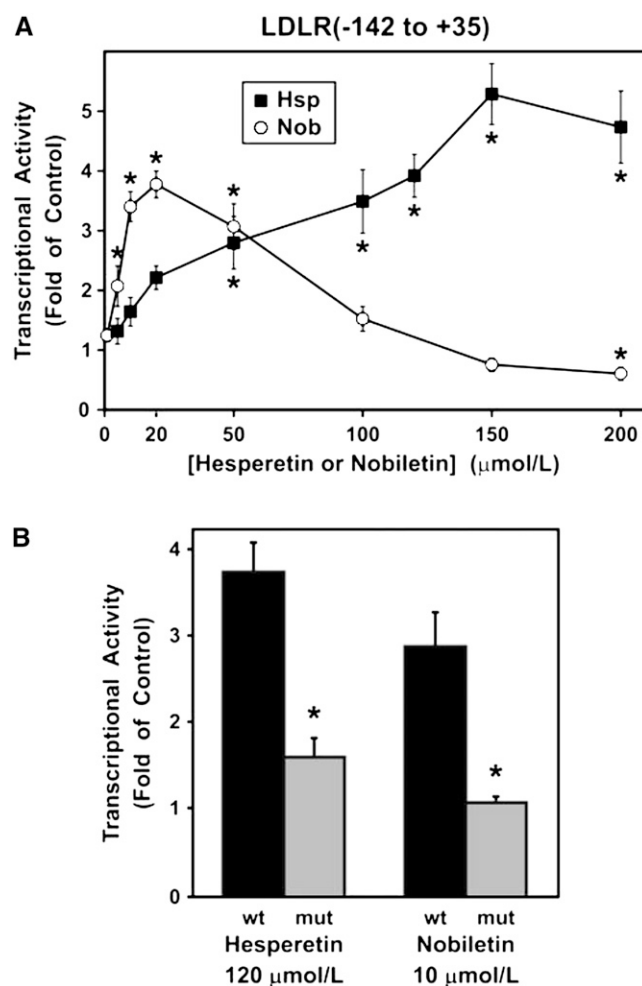


FIGURE 5 Dose effects of 24-h treatment with hesperetin or nobiletin on transcriptional activity of the LDLR promoter sequence from -142 to $+35$ (A) and reduction of flavonoid responsiveness by mutation of the SRE (B). Luciferase values were normalized and expressed as a fold of the corresponding untreated sample as described in the Online Supporting Material. Error bars are \pm SEM. (A) Transfected HepG2 cells were treated with hesperetin ($n = 4$ –6 at each dose) or nobiletin ($n = 5$ –7 at each dose) over a range of concentrations. *Different from the untreated control, $P \leq 0.05$ (Dunnett's test). (B) The SREBP binding site was either the native sequence (wt) or mutated at a single nucleotide (mut) ($n = 4$). *Different between the mut and the wt constructs for each compound at $P \leq 0.0001$ (ANOVA).

demonstrated here for the first time, to our knowledge, that stimulation of LDLR gene transcription by citrus flavonoids is dependent on the binding site for SREBP.

The most straightforward mechanisms to account for stimulation of gene transcription through an SRE are to increase SREBP total amount, nuclear availability, or inherent activity. A previous study showed that total SREBP-1 rises in response to naringenin (24). However, both the SREBP-1a isoform and SREBP-2 can induce LDLR mRNA in transgenic mice (45–48) and LDLR gene transcription in HepG2 cells (47). We have preliminary data from mRNA microarray experiments that show that SREBP-2 mRNA levels increased within 2 h of hesperetin treatment, attaining ~100% increase after 6 h (data not shown). SREBP-1 mRNA, on the other hand, was increased only slightly after 6 h of treatment. The increase in SREBP-2 mRNA is likely due, at least in part, to stimulation of gene transcription.

Because SREBP-2 regulates transcription of its own gene (45,47), we propose that the likely initial step in activation of this gene is either an increase in nuclear localization of SREBP-2 or stimulation of intrinsic SREBP-2 activity. Either mechanism could be controlled by signal transduction pathways, which is in agreement with the general concept that flavonoids act predominantly as cellular signaling molecules (49). In support of a role for kinase cascades in citrus flavonoid action, enhancement of LDLR mRNA levels by naringenin was blocked when phosphatidylinositol 3-kinase (PI3K) activity was inhibited (24). We have preliminary evidence suggesting that hesperetin and nobiletin induction of LDLR mRNA levels are also dependent on PI3K activity, because both responses were reduced in the presence of the PI3K inhibitor, wortmannin (data not shown). There is precedent for involvement of kinases through PI3K effects on the transport of SREBP-2 out of the endoplasmic reticulum in response to low sterol conditions (50) and enhancement of inherent transcriptional activity of both SREBP-1a and -2 through direct phosphorylation by mitogen-activated protein kinase in response to insulin (51,52). Therefore, our future studies will analyze the role of signal transduction pathways in citrus flavonoid enhancement of SREBP-2 activity and translocation to the nucleus and the resulting stimulation of transcription of the SREBP-2 and LDLR genes.

Potential mechanisms underlying the inverted-U dose response to nobiletin. We are not aware of any other investigations that have analyzed a biphasic dose response to flavonoids at the molecular level, but positive and negative effects across a dose range have been reported for hyperoside, a flavonoid found in *St. John's wort* (53), and the general phenomenon of an inverted-U response is well known for a variety of other stimuli (54–56). One possible explanation for this duality is that different metabolites of nobiletin are responsible for different effects, because conversion of PMF to a variety of forms in the liver has been found in hamsters (16). However, genistein gave a similar pattern of repression at high doses in the transfection assay (although results at the mRNA level were erratic and more difficult to interpret). Thus, the phenomenon does not appear to be unique to PMF, suggesting that a more general mechanism is likely.

Because the LDLR gene construct containing only 142 bp of upstream DNA is capable of mediating negative regulation by high doses of nobiletin (Fig. 5), a feasible scenario is that the SRE mediates both aspects of the biphasic response, with activation of SREBP at low doses and inhibition at high doses (e.g. by affecting different signaling cascades). However, we are not aware of any precedents in the literature for such effects on

either SREBP-1 or -2. It is certainly possible that other transcription factors besides SREBP are affected by high doses of nobiletin. Little is known, however, about negative regulation of the LDLR gene. One case of repression has been reported, but the DNA sequence necessary for this effect was ~1500 nucleotides upstream (i.e. not within the promoter-proximal region of our small construct) and a specific transcription factor was not identified (57). Another case of repression occurred through inhibition of the level of a positive transcription factor, CCAAT/enhancer binding protein β , which binds to a site very close to the start site of transcription (58,59), so it is possible that high doses of PMF could affect this factor.

Parallels between flavonoid effects in HepG2 cells and animal models. In our LDLR gene transcription assays, hesperetin was more efficacious, based on its higher maximal stimulation of LDLR gene transcription, than nobiletin (5.3- to 7.5-fold vs. 3.0- to 3.8-fold). Nobiletin satisfies the classical definition of being more potent, i.e. achieving maximal induction at lower doses than for hesperetin (10–20 $\mu\text{mol/L}$ vs. 150–160 $\mu\text{mol/L}$). There is good agreement between this difference in potency at the molecular level and previously reported differences between citrus flavanones and PMF at the physiological level. In the *in vivo* studies using dietary supplementation in hypercholesterolemic hamsters, citrus PMF were more potent than citrus flavanones at reducing serum cholesterol and TG (16). The total concentration of PMF in the livers of these animals reached ~16–67 $\mu\text{mol/L}$, which is in good agreement with the low- to mid-micromolar concentrations of tangeretin found to be effective in inhibiting apoB secretion, as well as other processes related to cholesterol and TG handling in HepG2 cells (25). Here, we found the PMF nobiletin to have peak activity at even lower doses.

The relatively high concentrations of flavanones in oranges and grapefruit have focused attention on the potential healthful effects of these citrus flavonoids. Although rather high dietary doses are sometimes used to maximize the effects in animal studies, flavanone concentrations in the plasma have been measured at 0.2–6 $\mu\text{mol/L}$ in humans after 1-time ingestion of 0.5–1.0 L of citrus juice (28,29) and higher amounts of the flavonoids may accumulate in the liver compared with the plasma levels (60). These concentrations of individual flavanones are low compared with the levels we show are needed for activity in isolated liver cells, but it is certainly possible that the combinations of flavonoids and metabolites that are present in the liver after ingestion of natural juice could act synergistically, which is consistent with the observation that daily consumption of 1 or 2 citrus fruits or 200 mL of juice reduces plasma cholesterol levels in humans (4–6). However, a potential disadvantage of fruit or juice consumption is maintaining consistent and high daily ingestion. In addition, the calories associated with high dietary intake could increase cardiovascular disease risk through weight gain. PMF are present in citrus fruit and juice in lesser amounts than flavanones, but, because the PMF are active at much lower doses, attainment of effective *in vivo* concentrations of these compounds by dietary supplementation is more plausible. In fact, supplements containing citrus PMF have shown promising cardioprotective effects in humans (18), although our data at the molecular level suggest that beneficial effects could be lost at very high doses. It will be important to reveal the underlying mechanisms of both the positive and negative effects of flavonoids in the liver to understand the potential advantages and disadvantages of the different classes of compounds and to develop the citrus flavonoids to their full

potential as therapeutic agents for combating heart disease, diabetes, and other chronic diseases.

Note Added in Proof. The latest update of GenBank accession no. NM_000527 (version 3 dated April 20, 2008) yields LDLR mRNA sizes of 5262, 4656, and 3597 bases, due to addition of 75 bases at the 5' end, 13 bases at the 3' end, and a few internal insertions and deletions. However, the sizes in Fig. 2 from NM_000527.2 are consistent with the numbering of the transcriptional regulatory region of the LDLR gene (42).

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